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Specification as originally filed, with Application for Patent Serial No: **2,304,906,** on April 7, 2000, by **1411198 ONTARIO LIMITED**, for "13Hode, A Regulator of Vascular Biocompatibility and an Inhibitor of Cell Hyperplasia".

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Date

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13-HODE, A REGULATOR OF VASCULAR BIOCOMPATABILITY AND AN INHIBITOR OF CELL HYPERPLASIA.

BACKGROUND OF THE INVENTION

Field of the Invention

In general, this invention relates to the regulation of vascular endothelium biocompatability, as well as to the inhibition of vessel wall (VW) cell and other types of cell hyperplasia following vessel wall (VW) dysfunction and/or injury. Specifically, this invention relates to the pharmaceutical preparations of 13-hydroxyoctadeca-9Z, 11E-dienoic acid (13-HODE) and its use orally to restore vascular endothelial cell biocompatability, thereby rendering the vasculature less reactive to circulating blood constituents during acute pathophysiological responses and decreasing chronic hyperplasic cell responses during and/or following VW stimulation, dysfunction or injury.

Preamble

Cell cell interactions play a fundamental role in the genesis of most diseases including cardiovascular disease, cancer and metatasis, and infection and inflammation. Below, we have focussed on cardiovascular disease as a specific example to illustrate in detail the disadvantages and limitations of current antithrombotic therapies and the utility and advantages of the invention in such a disease state. We then refer to other disease states in

which this invention will be useful.

Cardiovascular Disease: Treatment and Prevention - State of the Art

Cardiovascular disease is a major cause of morbidity and death in Western societies. It is exacerbated by smoking, hyperlipidemia, hypertension and diabetes. Over the last 40 years, our society has taken multiple steps to reduce cardiovascular disease such as promoting a healthier lifestyle, particularly in regard to smoking and diet. Nonetheless, each year, there are > 600,000 percutaneous transluminal coronary angioplasty (PTCA) and surgically invasive procedures, e.g. coronary artery bypass grafting (CABG) in N. America alone, performed in cardiovascular disease patients to improve (cardio)vascular blood flow. While these procedures are beneficial to many patients, the benefits are finite and short-lived, and VW stenosis will reoccur (RITA Trial Participants. Coronary angioplasty versus coronary artery bypass surgery: The randomized intervention treatment of angina (RITA) trial. *Lancet* 341: 573-580, 1993; Kirklin JW et al. Summary of a consensus concerning death and ischemic events after coronary artery bypass grafting. *Circ* 79 (Suppl 1): I81-I91, 1989). For example, restenosis occurs in 25-30% of patients within 6 months of PTCA despite acute heparin treatment, followed by continuous aspirin (ASA) treatment $\pm$ oral anticoagulants throughout the 6 month post PTCA period. Heparin is given to accelerate thrombin inhibition by antithrombin III (ATIII), thereby preventing fibrinogen cleavage to fibrin and subsequent fibrin clot formation; ASA is given to acetylate platelet cyclooxygenase, thereby inhibiting thromboxane A_2 (TxA_2) synthesis which renders platelets less reactive to prothrombotic stimuli; an oral anticoagulant, e.g. coumadin, is given

to decrease the level of vitamin K-dependent procoagulants, thereby decreasing the amounts of procoagulant substrates available for thrombus formation. Thus, the current approach to treat cardiovascular disease is to *impair* platelet function and/or coagulation as a means to prevent (re)occurrence of heart and blood vessel disease. It does not repair the underlying defect, the latter of which if attempted, might return the patient to a normal healthier state.

The only approaches currently proposed to reverse cardiovascular disease, are the use of lipid lowering agents which decrease the risk of atheriosclerotic lesion formation, and gene therapy. The former approach also has provided some benefit, but again, it does not 'fix' the underlying problem. Gene therapy may, in fact, address the issue of repairing the underlying defect(s), but gene therapy for cardiovascular disease is still in its infancy, and not without the risk of complex side effects (Libby P, Ganz P. Restenosis revisited - new targets, new therapies. *N Engl J Med* 337(6):418-419, 1997).

We propose that a treatment process which corrects the underlying cause of the disease problem involving the regulation of blood cell/VW compatability *per se,* is a more effective approach not only to treat the disease, but also to prevent it onset.

Rationale for Regulating VW Biocompatability

In order to better understand the rationale for regulating VW biocompatability and its benefits over current antithrombotic treatment practices, I first have reviewed the rationale behind the current antithrombotic strategies and their obvious limitations. This, in turn, will highlight some insights which have lead me and my colleagues to propose the concept of regulating VW biocompatability and different and novel approach of using 13-HODE to treat

and prevent cardiovascular disease.

1. *Vessel Wall Stenosis & Occlusion:* The problem of vascular stenosis and subsequent occlusion is one of the most important of all medical problems and can produce a very wide range of diseases, the best known of which are coronary, cerebral and peripheral arterial blockage. It is, of course, very difficult and perhaps impossible to study the earliest development of such arterial blockages in humans. People who feel healthy are not inclined to submit to invasive procedures which, in turn, may detect the onset of the disease before it manifests clinical symptoms. However, it is generally accepted that the processes of restenosis after an artery has been cleared or partially cleared of the occlusive material by a procedure such as angioplasty, is likely in many aspects to be similar to the processes involved in the original development of the problem. Vascular restenosis and occlusion after angioplasty or after vessel wall injury is therefore widely used as a model of the whole series of events involved in both primary and secondary arterial occlusion.

Vascular restenosis is thought to occur as a result of a combination of intimal smooth muscle cell (SMC) proliferation, SMC synthesis and secretion of extracellular matrix, and VW remodelling (Schwartz SM et al. The intima. Soil for atherosclerosis and restenosis. *Circ Res* 77: 445-465, 1995; Strauss BH et al. Extracellular matrix remodelling after balloon angioplasty in a rabbit model of restenosis. *Circ Res* 75: 650-658, 1994; Chervu A, Moore WS. An overview of intimal hyperplasia. *Surg* 171: 433-447, 1990; Bocan TMA, Guyton JR. Human aortic fibrolipid lesions: progenitor lesions for fibrous

plaques, exhibiting early formation of the cholesterol-rich core. *Am J Pathol* 120: 193-198, 1985). SMC proliferation *per se*, occurs in response to the mitogenic effects of thrombin generated at the time of VW injury, to platelet-derived growth factor (PDGF) secreted by platelets which adhere at the site of VW injury, and to mitogens secreted by activated endothelial cells (Bocan TMA, Guyton JR. Human aortic fibrolipid lesions: progenitor lesions for fibrous plaques, exhibiting early formation of the cholesterol-rich core. *Am J Pathol* 120: 193-198, 1985; Chen LB, Buchanan JM. Mitogenic activity of blood components. 1. Thrombin and prothrombin. *Proc Natl Acad Sci USA*. 72:131-135, 1975; Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* 362:801-809, 1993; Bretschneider E et al. Thrombin but not thrombin receptor activating peptide is mitogenic for coronary artery smooth muscle cells. *Thromb Res* 87 (5):493-497, 1997; Fischman DL et al. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. *N Engl J Med* 331:496-501, 1994; Grandaliano G et al. Thrombin regulates PDGF expression in bovine glomerular endothelial cells. *J Am Soc Nephrol* 9: 583 - 589, 1998; Stouffer GA et al. β_3 integrins are upregulated after vascular injury and modulate thrombospondin- and thrombin-induced proliferation of cultured smooth muscle cells. *Circ* 97:907-915, 1998).

Polymorphonuclear leukocytes (PMNs) and monocytes/macrophages also invade the VW injury site, activating both coagulation and platelets, thereby augmenting the hyperplasia process (Alexander RW. Inflammation and coronary artery disease. *N Engl J Med* 331:468-469, 1994; Mallory GA et al. The speed of healing of myocardial infarction: a study of the pathologic anatomy in 72 patients. *Am Heart J* 18:647-671, 1939; Mehta JL et al. Interactive role of infection, inflammation and traditional risk factors in atherosclerosis and coronary artery disease. *J Am Coll Cardiol* 31:1217-1225, 1998). Moreover, invading monocytes differentiate into macrophages, ingest lipids, calcium and other blood-derived constituents, thereby forming a more complex atheriosclerotic plaque

(Chervu A, Moore WS. An overview of intimal hyperplasia. *Surg* 171: 433-447, 1990; Bocan TMA, Guyton JR. Human aortic fibrolipid lesions: progenitor lesions for fibrous plaques, exhibiting early formation of the cholesterol-rich core. *Am J Pathol* 120: 193-198, 1985). Thus, there is a multiplicity of cell cell interactions which trigger and sustain intimal hyperplasia and subsequent restenosis (Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* 362:801-809, 1993; Schwartz RS. Pathophysiology of restenosis: interaction of thrombosis, hyperplasia, and/or remodeling. *Am J Cardiol* 81 (7A):14E-17E, 1998; Cicala C, Cirino G. Linkage between inflammation and coagulation: an update on the molecular basis of the crosstalk. *Life Sciences* 62 (20):1817-1824, 1997).

All of these events involve the interactions of blood components with the VW, which under 'healthy conditions' occur in response to injury and infection - but do not lead to (cardio)vascular disease. However, when these blood component/VW interactions are exaggerated such as with induced SMC proliferation, platelet/fibrin thrombus formation and VW hyperplasia, (cardio)vascular disease is initiated.

2. *VW Injury, Repair and Remodelling*: Recent studies debate the relative contributions of intimal VW hyperplasia *per se* versus VW remodelling after injury, to subsequent VW restenosis in the clinical setting. Lafont, Post, Mintz *et al* argue that VW remodelling associated with internal elastic lamina dilation or constriction, contributes more to restenosis after PTCA than intimal hyperplasia (Lafont A et al. Restenosis after experimental angioplasty: intimal, medial and adventitial changes associated with constrictive remodeling. *Circ* 76:996-1002, 1995; Post MJ et al. The relative importance of arterial remodeling compared with intimal hyperplasia in lumen renarrowing after balloon angioplasty: a study in the normal rabbit and in the hypercholesterolemic Yucatan micropig. *Circ*

89:2816-2821, 1994). The results of the *Benestent* and STRESS studies are said to be consistent with that argument since increasing the coronary artery diameter with a stent, decreases the need for revascularization (Fischman DL et al . A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. *N Engl J Med* 331:496-501, 1994; Grandaliano G et al. Thrombin regulates PDGF expression in bovine glomerular endothelial cells. *J Am Soc Nephrol* 9: 583 - 589, 1998. Coats *et al* agree since there is more SMC-derived collagen (and presumeable more SMCs) in non-stenosed VWs than in stenosed VWs (Coats WD et al. Collagen content is significantly lower in restenotic versus nonrestenotic vessels after balloon angioplasty in the atherosclerotic rabbit model. *Circ* 95:1293-1300, 1997). McGee MP et al. Specific regulation of procoagulant activity on monocytes. *J Biol Chem* 270 (44):26109-226115, 1995.). The opposite might be expected if hyperplasia was the predominate cause for restenosis . Coats *et al* suggested that the failure of our current antithrombotic therapy to inhibit restenosis as effectively as expected, is because that therapy focuses predominately on inhibiting SMC proliferation. These conclusions, however, do not consider the heterogeneity of proliferating SMCs and their capacity to synthesize various matrices (Frid MG et al. Smooth muscle cell heterogeneity in pulmonary and systemic vessels. *Arterioscler Thromb Vasc Biol* 17:1203-1209, 1997), or the fact that SMC collagen synthesis is affected by the presence (or absence) of the endothelium. Specifically, endothelial cells inhibit SMC protein synthesis, particular type III collagen (Myers PR, Tanner MA. Vascular endothelial cell regulation of extracellular matrix collagen: role of nitric oxide. *Arterioscler Thromb Vasc Biol* 18:717-722, 1998). The opposite is not true. We also found that the extracellular matrix within a hyperplastic intima of a 1st injury VW, is rich in elastin while

the extracellular matrix within the hyperplastic intima of a 2nd injury VW, is rich in collagen (Buchanan MR, Brister SJ. Inhibition of chronic vessel wall (re)stenosis with acute thrombin inhibition: Relative effects of heparin and dermatan sulphate. *Thromb Res* 91: 157 - 167, 1998), consistent with other studies (Capron Let al. Repeated balloon injury of rat aorta: a model of neointima with attenuated inhibition by heparin. *Arterioscler Thromb Vasc Biol* 17:1649-1656, 1997.). Moreover, the clinical studies cited above were performed with patients who also required a stent due to the complex nature of their lesions. The restenosis rate in those patients is > 4 x's the restenosis rate in PTCA patients who do not require a stent (Antoniucci D et al. Restenosis after coronary stenting in current clinical practice. *Am Heart J* 135:510-518, 1998). The treatment of PTCA patients who require a stent also differs significantly from the treatment of PTCA patients who do not require a stent (Antoniucci D et al. Restenosis after coronary stenting in current clinical practice. *Am Heart J* 135:510-518, 1998). These differences are likely to affect subsequent outcome, both at the basic and the clinical end point levels. It is more likely that the relative roles of SMC hyperplasia and VW remodelling in restenosis varies depending on the type of injury and the type of the antithrombotic therapy use.

3. Blood Cell/Injured Vessel Wall Interactions: Normally, the VW is nonthrombogenic and, therefore, biocompatable with the circulating blood. When the VW is injured, it becomes highly thrombogenic. Injured veins and arteries express tissue factor in both their media and intima. This expression increases over time after injury. Tissue factor expression is minimal in uninjured VWs (Channon KM et al. Modulation of tissue factor protein expression in experimental

ven us bypass grafts. *Arterioscler Thromb Vasc Biol* 17; 1313-1319, 1997). Tissue factor expression is enhanced further by PMNs and/or monocyte/macrophages which invade the injury site. This enhancement is dependent on PMN and/or monocyte/macrophage CD18 integrin expression (Channon KM et al. Modulation of tissue factor protein expression in experimental venous bypass grafts. *Arterioscler Thromb Vasc Biol* 17; 1313-1319, 1997; McGee MP et al. Specific regulation of procoagulant activity on monocytes. *J Biol Chem* 270 (44):26109-226115, 1995). VW tissue factor expression activates prothrombin which is widely distributed throughout VW tissue rich in SMCs (McBane RD et al. Tissue prothrombin: universal distribution in smooth muscle. *Arterioscler Thromb Vasc Biol* 17:2430-2436, 1997). Thrombin upregulates endothelial cell PDGF receptor expression, thereby facilitating SMC proliferation (Grandaliano G et al. Thrombin regulates PDGF expression in bovine glomerular endothelial cells. *J Am Soc Nephrol* 9: 583 - 589, 1998; DiCorleto PE, Bowen-Pope DF. Cultured endothelial cells produce a platelet-derived growth factor-life protein. *Proc Natl Acad Sci USA* 80: 1919 - 1923, 1983), and platelet activation. Activated platelets secrete TxA_2 (which is vasoconstrictive), PDGF (which is mitogenic for SMCs) and procoagulants which exacerbate coagulation (Pakala R et al. Effect of serotonin, thromboxane A_2, and specific receptor antagonists on vascular smooth muscle cell proliferation. *Circ* 96:2280-2286, 1997). Platelet-related factor Xa/Va activity bound to the injured VW, also renders it highly thrombogenic. This latter effect persists for > 96 hours (Ghigliotti G et al. Prolonged activation of prothrombin on the vascular wall after arterial injury. *Arterioscler Thromb Vasc Biol* 18:250-257, 1998).

Thus, the multiplicity of these events suggests that there is a sound rationale for using an antithrombotic therapy which targets coagulation, platelet function and inflammation.

However, there also is sound rationale for using a therapy which targets VW thrombogenicity *per se.* To date, this latter approach is virtually nonexistant.

4. Anticoagulant Therapy and VW Hyperplasia: A number of studies demonstrate that heparin can inhibit experimentally-induced SMC proliferation *in vitro* and *in vivo* (Castellot JJ Jr et al. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. *J Cell Physiol* 58: 315-320, 1984; Clowes AW, Clowes MM. Kinetics of cellular proliferation after arterial injury: heparin inhibits rat smooth muscle mitogenesis and migration *Circ Res* 58: 839-845, 1986; Ferrell M et al. A dilemma for the 1990's: Choosing appropriate experimental animal models for the prevention of restenosis. *Circ* 85: 1630-1631, 1992; Hanke H et al. Inhibition of cellular proliferation after experimental balloon angioplasty by low-molecular-weight heparin. *Circ* 85: 1548-1556, 1992). This suggests that heparin should prevent SMC hyperplasia and subsequent restenosis clinically. However, restenosis occurs clinically despite heparin treatment. It is now recognized that thrombin is protected from inhibition by ATIII and the acceleration of that effect by heparin when thrombin binds to fibrin or other constituents on the injured VW surface (Okwusidi JI et al. Fibrin moderates the catalytic action of heparin but not that of dermatan sulfate on thrombin inhibition in human plasma. *J Lab Clin Med* 117: 359-364, 1991; Okwusidi JI et al. *In vivo* catalysis of thrombin inhibition by antithrombin III and heparin co-factor II and antithrombotic effect: Differential effects of dermatan sulfate and unfractionated heparin. *Thromb Haemorrh Dis* 2: 17-23, 1990; Hogg PJ, Jackson CM. Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: Implications for heparin efficacy. *Proc Natl Acad Sci USA* 86: 619-623, 1989; Bar-Shavit R, Eldor A. Vlodavsky I. Binding of thrombin t subend thelial extracellular matrix. *J Clin Invest* 84: 1098-1104, 1989). Moreover, the

surface-bound thrombin remains active, contributing to systemic hypercoagulation despite anticoagulant therapy (Ghigliotti G et al. Prolonged activation of prothrombin on the vascular wall after arterial injury. *Arterioscler Thromb Vasc Biol* 18:250-257, 1998; Brister SJ et al. Thrombin generation during cardiac surgery. Is heparin the ideal anticoagulant? *Thromb Haemost* 70: 259-263, 1993; Wells J et al. Thrombin generation in patients undergoing carotid endarterectomy: Implications in acute vessel wall closure and antithrombotic therapy. *Thromb Res* 75: 419-426, 1994; Gill JB et al. Thrombin generation post PTCA following cessation of heparin infusion. *Can J Cardiol* 9(E): 84E, 1993). Consequently, surface-bound thrombin can activate platelets, SMC proliferation and further coagulation unchecked. There also is evidence that SMCs which proliferate in response to repeated injury, are less sensitive to the heparin treatment than SMCs which proliferate in response to a first injury (Capron L et al. Repeated balloon injury of rat aorta: a model of neointima with attenuated inhibition by heparin. *Arterioscler Thromb Vasc Biol* 17:1649-1656, 1997; Geary RL et al. Failure of heparin to inhibit intimal hyperplasia in injured baboon arteries: the role of heparin-sensitive and -insensitive pathways in the stimulation of smooth muscle cell migration and proliferation. *Circ* 91: 2972-2981, 1995).

5. Antiplatelet Therapy and Hyperplasia: There is little evidence that antiplatelet therapy *per se* reduces SMC hyperplasia. Clearly, ASA is beneficial in reducing the risks of stroke, myocardial infarction and transient ischemic attacks in patients with a variety of cardiovascular diseases (Aspirin Trialists' Collaboration. Collaborative overview of randomized trials of antiplatelet therapy. II. Maintenance of vascular graft or arterial patency by antiplatelet therapy. *Br Med J* 308: 159-168, 1994). However, the overall risk reduction with ASA, is only ≈ 25% (Aspirin Trialists' Collaboration. Collaborative overview of randomized trials of antiplatelet therapy. II. Maintenance of vascular graft or arterial patency by antiplatelet therapy. *Br Med J* 308: 159-168, 1994). While this risk

reduction is *statistically* significant, the reduction is modest at best.

Also, ASA may benefit only certain subgroup of patients (Buchanan MR, Brister SJ. Individual variation in the effects of ASA on platelet function: Implications for the use of ASA clinically. *Can J Cardiol* 11: 317-321, 1995; Grotemeyer K-H et al. Two year follow up of aspirin responders and non-responders. A pilot study including 180 post stroke patients. *Thromb Res* 71: 397-403, 1993; Grotemeyer KH. Effects of acetylsalicylic acid in stroke patients: Evidence of nonresponders in a subpopulation of treated patients. *Thromb Res* 63: 587-593, 1991). This may be due, in part, to the wide variation in platelet responsiveness to assorted stimuli after ASA ingestion (Mueller MR et al. Variable platelet response to low-dose ASA and the risk of limb deterioration in patients submitted to peripheral arterial angioplasty. *Thromb Haemost* 78:1003-1007, 1997). The effect of ASA is also finite and has little benefit after 2 years (Aspirin Trialists' Collaboration. Collaborative overview of randomized trials of antiplatelet therapy. II. Maintenance of vascular graft or arterial patency by antiplatelet therapy. *Br Med J* 308: 159-168, 1994).

Alternate antiplatelet agents which block the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor have been proposed as superior alternates to ASA. The EPILOG study demonstrated that blocking the GPIIb/IIIa receptor with c7E3 decreases acute ischemic complications in patients undergoing PTCA (The EPILOG Investigators. Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. *N Engl J Med* 336:1689-1696, 1997). Similar results were obtained in the PRISM study using Aggrastat, a non-peptide GPIIb/IIIa antagonist (The Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators. A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. *N Engl J Med* 338:1498-1505, 1998). It also has been suggested that the short $t^1/_2$ of these

compounds may circumvent any bleeding side effect as compared to ASA. However, the bleeding issue still remains controversial. More importantly, like with aspirin, there is little clinical evidence to suggest that long-term hyperplasia is inhibited by these compounds.

Given recent studies, it is not surprising that platelet function inhibitors have little effect on preventing hyperplasia and restenosis. Specifically, Sirois *et al* made animals thrombocytopenic and then injured their arteries. Thrombocytopenia was sustained for short or long periods of time, and then their platelet counts were restored to normal levels. While the onset of VW hyperplasia was delayed in the long-term thrombocytopenic animals, the potential for SMC proliferation was not inhibited at all. Thus, medial SMC PDGR-β receptor expression was upregulated in all animals despite their being or not being thrombocytopenic. As a result, when the platelet count was restored to normal, SMC proliferation and subsequent intimal hyperplasia were initiated (Sirois MG et al. Rat arterial wall retains myointimal hyperplastic potential long after arterial injury. *Circ* 96:1291-1298, 1997). These data not only emphasize the need to regulate acute platelet/VW interactions to inhibit chronic intimal hyperplasia, but also suggest that platelet inhibition alone for any finite period of time is unlikely to have a lasting effect.

Limitations with the Current Antithrombotic Therapies

While all of the studies cited above, both experimental and clinical, clearly indicate clinical benefits with the varied approaches to attenuate the different stages in the development of atherosclerosis, none of these approaches prevent disease onset or facilitate

disease regression. Moreover, all of the therapeutic approaches mentioned above act indirectly by compromising coagulation, platelet function and/or injured vessel wall repair. As a result, all patients receiving any form of the currently recommended antithrombotic therapies, are rendered hemostatically dyfunctional, and therefore, at a significant hemorrhagic risk. Thus, there is a clear need for a better antithrombotic approach which leads to the prevention and/or reversal of vascular disease, and which achieves these effects without any adverse side effects.

13-HODE, VW Biocompatability and Hyperplasia

The concept of preventing VW hyperplasia by altering VW biocompatability has not been considered directly, except perhaps, from the perspective of reducing fat and cholesterol intake in an attempt to reduce VW lipid accumulation and fatty streak formation on the VW. Most attempts have focussed more on the isolation and recombinant synthesis and subsequent utilization of VW constituents to alter blood component properties. For example, there is both experimental and clinical data to suggest that endothelial cell-derived nitric oxide, tissue plasminogen activator and prostacyclin are useful in the treatment of patients at risk of acute thromboembolic events (Gershlick AH et al. Failure of epoprostenol (prostacyclin, PGI_2) to inhibit platelet aggregation and to prevent restenosis after coronary angioplasty: results of a randomized placebo controlled trial. *Br Heart J* 71: 7-12, 1994; Zerkowski H-R et al. Endothelial damage of the venous graft in CABG: influence of solutions used for storage and rinsing on endothelial function. *Eur J Cardio-Thoracic Surg* 7: 376-382, 1993; The GUSTO Investigators. An international rand mized trial

c mparing four thrombolytic strategies for acute myocardial infarction. *N Engl J Med* 329: 673-682, 1993). Their effects, like the antiplatelet and anticoagulant therapies, target platelet function, vessel wall calibre and thrombolysis, thereby compromising hemostasis and coagulation. Moreover, it should be noted that all of these are only produced by the VW following injury or activation, and have little effect on regulating the innate biocompatable properties of a healthy, injured or diseased VW *per se*. Given the limited success in the current antithrombotic therapies in preventing acute thrombus formation and atherogenesis and ensuing atherosclerosis, my colleagues and I refocussed our research endeavours to identify any endogenous VW factor(s) which might influence the biocompatability of the vascular endothelium. To that end, we discovered that the healthy vessel wall synthesizes 13-HODE. It is now recognized that 13-HODE is produced in various cells and tissues in the body, particularly by vascular endothelial cells and by dermal epithelial cells. 13-HODE is formed by the action of an enzyme known as 15-lipoxygenase on the dietary essential fatty acid, linoleic acid. The first step is oxidation of the linoleic acid to give 13-hydroperoxyoctadeca-9Z, 11E-dienoic acid (13-HpODE). This is then reduced to 13-HODE. 13-HODE is an important signal transduction molecule which is short-lived and whose synthesis is activated by a variety of different stimuli (Buchanan MR et al. 13-Hydroxy-octadecadienoic acid is the vessel wall chemorepellant factor, LOX. *J Biol Chem* 260: 16056-16059, 1985; Haas TA et al. Cyclic AMP regulation of endothelial cell triglyceride turnover, 13-hydroxyoctadecadienoic acid (13-HODE) synthesis and endothelial cell thrombogenicity. *Biochim Biophys Acta* 1031: 174-178, 1990; Weber E et al. Relationship between vessel wall 13-HODE synthesis and vessel wall thrombogenicity following injury. Influence f

salicylate and dipyridamole treatment. *Thromb Res* 57: 383-392, 1990; Bertomeu M-C et al. Selective effects of dietary fats on vascular 13-HODE synthesis and platelet/vessel wall interactions. *Thromb Res* 59: 819-830, 1990; Brister SJ et al. 13-HODE synthesis in internal mammary arteries and saphenous veins: Implications in cardiovascular surgery. *Adv Prost Thromb Leuko Res* 21: 667-670, 1990; Buchanan MR, Bastida E. Endothelium and underlying membrane reactivity with platelets, leukocytes and tumor cells: regulation by the lipoxygenase-derived fatty acid metabolites, 13-HODE and HETE's. *Med Hypothesis* 27: 317 - 325, 1988; Cho Y, Ziboh VA. Incorporation of 13-HODE into epidermal ceramides and phospholipids: phospholipase C-catalyzed release of 13-HODE-containing diaglycerol. *J Lipid Res* 35: 255 - 262, 1994; Mari I. Upregulation of nuclear PKC and MAPkinase during hyperproliferation of guinea-pig epidermis: modulation of 13-HODE. *Cell Signalling* 10: 143 - 149, 1998; Kang L-T *et al.* Novel membrane target proteind for lipoxygenase-derived mono (5) hydroxy fatty acids. *Biochim Biophys Acta* 1438: 388 - 398, 1999; Pongracz J, Lund JM. The lipoxygenase product 13-HODE is a selective inhibitor of classical PKC isoenzymes. *Biochem Biophys Res Comm* 256: 269 - 272 B, 1999; Friedrichs et al. 13-HPODE and 13-HODE modulate cytokine-induced expression of endothelial cell adhesion molecules differently. *BioFactors* 9: 61 - 72, 1999; Cho Y, Ziboh VA. 13-hydroxyoctadecadienoic acid reverses epidermal hyperproliferation via selective inhibition of protein kinase C-beta activity. *Biochem Biophys Res Comm* 201: 257 - 265, 1994). Many of the effects of 13-HODE are mediated by inhibition of protein kinases (PK), particularly PKC and mitogen-activated PK (MAP kinase).

13-HODE which is an oily liquid can be incorporated in much the same way as its parent fatty acid, linoleic acid, into a range of complex molecules including phospholipids and triglycerides (Spiteller G. Linoleic acid peroxidation, the dominant lipid peroxidationprocess in low density lipoprotein and its relationship to chronic diseases. *Chem Physics Lipids*: 95: 105 - 162. 1998; Fang

X et al. 13-HODE incorporation and conversion to novel products by endothelial cells. *J Lipid Res* 40: 699 - 707, 1999). 13-HODE which is not incorporated into complex lipids is rapidly metabolized by hydrogenation and beta-oxidation (Bronstein JC, Bull AW. The correlation between 13-HODE dehydrogenase and intestinal cell differentiation. *Prostaglandins* 46: 387 - 395. 1993; Hecht, Spiteller G. Linoleic acid peroxidation products are metabolized by hydrogenation in porcine liver tissue. *Eur Mass Spectrometry* 4: 393 - 399, 1998).

Almost all of the studies designed to investigate the effects of 13-HODE involve measuring the effects of altering endogenous 13-HODE production or by adding exogenous 13-HODE (in various forms) to cultured cells *in vitro*. In the past, there have been few studies which measure the effects of 13-HODE when given orally or parenterally to animals or humans. This limitation has been due, in part, to the difficults of making large quantities of 13-HODE and its availability to the scientific community. Consequently, the amount of 13-HODE needed for *in vivo* studies has been extremely expensive. Second, generally it has been believed that 13-HODE is unstable and readily metabolized, like many signal molecules. As such, it has been thought that it would be a waste of time and money to perform studies involving the oral administration of 13-HODE since none of the orally-administered material would be expected to reach its target site of action.

However, there are a few studies which suggest that orally-administered 13-HODE has biological relevent effects *in vivo*. Streber's patent describes the use of 13-HODE and other related fatty acids to inhibit aromatase enzymes which convert androgens to estrogens. The purpose of the treatment is to act on any disease which is induced by estrogen such as

breast cancer and possibly some types of benign prostatic hyperplasia. However, it should be noted that all of the evidence provided in Streber's patent is based on data obtained *in vitro*. There are no experiments which demonstrate that that invention actually works *in vivo*. Moreover, Streber does not provide any details regarding the methods of administration nor any practical details as to how the materials might be formulated (although it is stated that 'tablets or capsules or dragees' may be used). Finally, the daily dose specified ranges from 100 to 1,000 mg, preferably in the 200 to 500 mg range. (Streber AS. Hydroxy-octadecadienoic acid for the treatment of estrogen-dependent disease. *US Patent # 5,102,912*, April 1992).

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The only study known to us which actually describes any experiments whereby a hydroxy derivative of linoleic acid has been administered orally outside of our own experiences (discussed below), is that of Kaminakai *et al* (Japanese patent, #7-291862, November 7, 1995). However, they only mention 13-HODE in passing. The actual hydroxy derivative of linoleic acid manufactured for patent use in their experiments, is a different fatty acid; namely, 9-hydroxy-10(E)-12(Z) octadecadienoic acid (9-HODE) which is described in the NMR spectrum shown in Figure 2 of their patent, and which is stated in the text to be the material actually manufactured and studied in their experiments. These experiments involved the use of 9-HODE given orally to influence the action of Sarcoma 180 tumors implanted
20 in the abdominal cavity of mice. They report an inhibitory effect on the rate of tumour growth, but the minimum effective dose required is 55 mg/kg/day.

The only 13-HODE studies which focus specifically on altering VW biocompatability to prevent thrombogenesis have been reported by us. Our earlier studies demonstrate that healthy VW cells continuously turn over linoleic acid; i.e. at a time when the endothelium is nonthrombogenic or biocompatable with the circulating blood (Buchanan MR et al. 13-Hydroxy-octadecadienoic acid is the vessel wall chemorepellant factor, LOX. *J Biol Chem* 260: 16056-16059, 1985). Intracellular linoleic acid is metabolized to 13-HODE via the lipoxygenase pathway (Haas TA et al. Cyclic AMP regulation of endothelial cell triglyceride turnover, 13-hydroxyoctadecadienoic acid (13-HODE) synthesis and endothelial cell thrombogenicity. *Biochim Biophys Acta* 1031: 174-178, 1990).

We also reported that:

i) VW cell thrombogenicity varies inversely with VW 13-HODE levels in both animals and humans (Weber E et al. Relationship between vessel wall 13-HODE synthesis and vessel wall thrombogenicity following injury. Influence of salicylate and dipyridamole treatment. *Thromb Res* 57: 383-392, 1990; Bertomeu M-C et al. Selective effects of dietary fats on vascular 13-HODE synthesis and platelet/vessel wall interactions. *Thromb Res* 59: 819-830, 1990; Brister SJ et al. 13-HODE synthesis in internal mammary arteries and saphenous veins: Implications in cardiovascular surgery. *Adv Prost Thromb Leuko Res* 21: 667-670, 1990; Buchanan MR, Brister SJ. Increasing vessel wall (VW) 13-HODE metabolism with dietary fatty acid supplements in patients undergoing cardiac surgery decreases VW reactivity to platelets. *Can J Cardiol* 10 (Suppl C): 67C, 1994);

ii) increasing 13-HODE in both rabbit and human VWs before injury decreases platelet/VW interactions following injury (Weber E et al. Relationship between vessel wall 13-HODE synthesis and

vessel wall thrombogenicity following injury. Influence of salicylate and dipyridam le treatment. *Thromb Res* 57: 383-392, 1990; Bertomeu M-C et al. Selective effects of dietary fats on vascular 13-HODE synthesis and platelet/vessel wall interactions. *Thromb Res* 59: 819-830, 1990; Buchanan MR, Brister SJ. Increasing vessel wall (VW) 13-HODE metabolism with dietary fatty acid supplements in patients undergoing cardiac surgery decreases VW reactivity to platelets. *Can J Cardiol* 10 (Suppl C): 67C, 1994); and

iii) 13-HODE downregulates the ability of the vitronectin receptor to recognize its ligands, thereby decreasing its adhesivity for vitronectin, fibronectin and fibrin(ogen) (Buchanan MR et al. Regulation of endothelial cell/ and platelet/receptor-ligand binding by the 12- and 15-lipoxygenase monohydroxides, 12-, 15-HETE and 13-HODE. *Prost Leuko Essential Fatty Acids* 58: 339-346, 1998).

However, it is important to emphasize that in all of these studies, the aim was always to raise the level of endogenous production of 13-HODE by manipulating its endogenous synthesis or breakdown. It never occurred to us that it might be valuable to manipulate vessel wall 13-HODE levels by the exogenous administration of 13-HODE. This is clearly demonstrated by our next series of experiments involving the administration of Persantine (dipyridamole) which is a phosphodiesterase inhibitor and which we thought might regulate endogenous 13-HODE metabolism.

These studies suggested to us that an antithrombotic therapy which involves increasing VW 13-HODE levels, should decrease SMC hyperplasia. To test that possibility, we treated rabbits with Persantine (1 mg/kg/day for 7 days) before a 1st or a 2 nd VW injury, and then we measured intimal SMC hyperplasia 4 weeks later. Persantine was given on the

basis that it inhibits phosphodiesterase, thereby increasing VW cAMP levels (Weber E et al. Relationship between vessel wall 13-HODE synthesis and vessel wall thrombogenicity following injury. Influence of salicylate and dipyridamole treatment. *Thromb Res* 57: 383-392, 1990;Haas TA et al. Cyclic AMP regulation of endothelial cell triglyceride turnover, 13-hydroxyoctadecadienoic acid (13-HODE) synthesis and endothelial cell thrombogenicity. *Biochim Biophys Acta* 1031: 174-178, 1990). Increasing VW cAMP increased VW linoleic acid turnover and subsequent VW 13-HODE synthesis, which, in turn, was associated with decreased platelet/VW interactions at the time of injury (Weber E et al. Relationship between vessel wall 13-HODE synthesis and vessel wall thrombogenicity following injury. Influence of salicylate and dipyridamole treatment. *Thromb Res* 57: 383-392, 1990). The Persantine treatment inhibited SMC hyperplasia 4 weeks after VW injury. Platelet function in these animals, was unchanged. These studies are consistent with the possibility that decreasing VW thrombogenicity by increasing VW 13-HODE at the time of injury will attenuate long-term intimal hyperplasia and subsequent VW restenosis. Moreover, this approach did not compromise normal hemostasis and coagulation like the currently used clinical approaches do.

13-HODE, Anti-inflammatory Therapy and VW Hyperplasia

Inflammation has been recognized as an integral part of the thrombotic process as early as 1939 (Mallory GA et al. The speed of healing of myocardial infarction: a study of the pathologic anatomy in 72 patients. *Am Heart J* 18:647-671, 1939), yet it is not considered in the rationale for our current antithrombotic therapies. However, there is convincing evidence that attenuating

certain inflammatory responses provide a significant benefit. For example, monocytes/macrophages and PMNs express the integrin CD11/CD18 (ICAM), and they release cytokines when activated (Peracchia R et al. cAMP involvement in the expression of MMP-2 and MT-MMP1 metalloproteinases in human endothelial cells. *Arterioscler Thromb Vasc Biol* 17:3185-3190, 1997; Yasukawa H et al. Inhibition of intimal hyperplasia after balloon injury by antibodies to intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1. *Circ* 95:1515-1522, 1997; Turek JJ et al. Modulation of macrophage cytokine production by conjugated linoeic acids is influenced by dietary n-6:n-3 fatty acid ratio. *J Nutr Biochem* 9: 258 - 266, 1998),which, in turn, stimulate i) β_3 integrin expression in other cells such as platelets, endothelial cells and SMCs (Blanks J E et al. Stimulation of P-selectin glycoprotein ligand-1 on mouse neutrophils activates β_2-integrin mediated cell attachment to ICAM-1. *Eur J Immunol* 28:433-443, 1998; Golino P et al. Inhibition of leucocyte and platelet adhesion reduces neointimal hyperplasia after arterial injury. *Thromb Haemost* 77(4):783-788, 1997); ii) tissue factor activation (McGee MP et al. Specific regulation of procoagulant activity on monocytes. *J Biol Chem* 270 (44):26109-226115, 1995); and iii) PDGF expression (Rubin P et al. Cellular and molecular mechanisms of radiation inhibition of restenosis. Part I: Role of the macrophage and platelet-derived growth factor. *Int J Radiation Oncology Biol Phys* 40: 929 - 941, 1998; Panek RL et al. PDGF receptor protein tyrosine kinase expression in the balloon-injured rat carotid artery. *Thromb Vasc Biol* 17:1283-1288, 1997). Lipid fractions derived from platelets augment these responses by inducing monocyte/macrophage differentiation and growth (Ammon C et al. Platelets induce monocyte differentiation in serum-free coculture. *J Leukoc Biol* 63:469-476, 1998). Macrophages interacting with the injured vessel wall, accumulate lipid, leading to the formation of a more complex atherosclerotic lesion (Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* 362:801-809, 1993; Post MJ et al.

The relative imp rtance of arterial remodeling compared with intimal hyperplasia in lumen renarrowing after balloon angioplasty: a study in the normal rabbit and in the hypercholesterolemic Yucatan micropig. *Circ* 89:2816-2821, 1994). Blocking monocyte/macrophage ICAM expression reduces VW hyperplasia significantly (Golino P et al. Inhibition of leucocyte and platelet adhesion reduces neointimal hyperplasia after arterial injury. *Thromb Haemost* 77(4):783-788, 1997; Nageh MR et al. Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 17:1517-1520, 1997; Natori S et al. Prostaglandin E_1 protects against ischemia reperfusion injury of the liver by inhibition of neutrophil adherence to endothelial cells. *Transplantation* 64 (11) 1514-1520, 1997). Others have found that radiation (^{90}Sr/Y or ^{192}Ir) at doses which selectively impair monocyte/macrophage function also decreases VW hyperplasia in both rodent and rabbit models (Rubin P et al. Cellular and molecular mechanisms of radiation inhibition of restenosis. Part I: Role of the macrophage and platelet-derived growth factor. *Int J Radiation Oncology Biol Phys* 40: 929 - 941, 1998; Panek RL et al. PDGF receptor protein tyrosine kinase expression in the balloon-injured rat carotid artery. *Thromb Vasc Biol* 17:1283-1288, 1997; Williams DO. Radiation vascular therapy: a novel approach to preventing restenosis. *Am J Cardiol* 81 (7A):18E-20E, 1998; Kipshidze N et al. Photoremodeling of arterial wall reduces restenosis after balloon angioplasty in an atherosclerotic rat model. *J Am Coll Cardiol* 31:1152-1157, 1998). In a preliminary clinical study, the SCRIPPS trial using endovascular radiation, the restenosis rate in 35 patients undergoing PTCA who also required a stent was 11%, significantly less than the 37% restenosis rate seen in comparable non-irradiated controls (Williams DO. Radiation vascular therapy: a novel approach to preventing restenosis. *Am J Cardiol* 81 (7A):18E-20E, 1998). These data provide direct evidence which suggests that altering inflammatory responses also affect intimal hyperplasia and subsequent VW restenosis.

Studies by me and my colleagues suggest that the culprit inflammatory cell is not the PMN. In fact, PMNs may attenuate the vessel wall thrombogenicity by providing a source of 13-HODE at the site of blood cell/VW interactions at the time of VW injury (Buchanan MR. Inhibition of thrombosis by leukocytes: role of endogenous fatty acid metabolites. *Sanofi Fdn Thromb Res Bull* 4: 14-22, 1989; Buchanan MR et al. Altering vessel wall fatty acid metabolism: A new strategy for antithrombotic treatment. *Sem Thromb Hemost* 19: 149-157, 1993). Others have argued that PMN-derived oxygen radicals promote ischemia-related damage (Shen J et al. Macrophage-mediated 15-lipoxygenase expression protects against atherosclerosis development. *J Clin Invest* 98:2201-2208, 1996), but this has not been linked to long-term hyperplasia. PMNs also secrete a nitric oxide-like factor which inhibits platelet function and vasoconstriction (Cerletti C et al. Polymorphonuclear leucocyte-dependent modulation of platelet function: relevance to the pathogenesis of thrombosis. *Pharmacol Res* 26 (3):261-268, 1992). Monocytes/macrophages, on the other hand, normally do not synthesize 13-HODE (Shen J et al. Macrophage-mediated 15-lipoxygenase expression protects against atherosclerosis development. *J Clin Invest* 98:2201-2208, 1996; Shen J et al. Transgenic rabbits with the integrated human 15-lipoxygenase gene driven by a lysozyme promoter: macrophage-specific expression and variable positional specificity of the transgenic enzyme. *FASEB J* 9:1623-1631, 1995). Interestingly however, Shen *et al* upregulated 15-lipoxygenase in differentiated macrophages and found that 13-HODE synthesis increased. This increase was associated with decreased macrophage lipid accumulation. Fan *et al* also found that macrophages enriched with linoleic and gamma linolenic acids (substrates for 13-HODE and PGE_1, respectively), stimulate intracellular SMC cAMP which, in turn, decreases SMC proliferation (Fan YY et al. Dietary *gamma*-linolenic

acid enhances mouse macrophage-derived prostaglandin E_1 which inhibits vascular smooth muscle cell proliferation. *J Nutr* 127:1765-1771, 1997). Finally, 13-HODE inhibits PAF (platelet activating factor)-induced PMN and monocyte/macrophage degranulation and ICAM expression (Cerletti C et al. Polymorphonuclear leucocyte-dependent modulation of platelet function: relevance to the pathogenesis of thrombosis. *Pharmacol Res* 26 (3):261-268, 1992), thereby preventing further integrin-dependent cell cell and cell ligand interactions.

Recent Studies with 13-HODE

The above data suggest that endogenous VW 13-HODE plays an important role in regulating VW biocompatability under both healthy and thrombogenic situations. If so, it suggests that 13-HODE is a useful antithrombotic agent. However, any progress in developing that concept has been thwarted by the lack or absence of any agent which would directly upregulate 13-HODE synthesis by VW cells, PMNs or other relevent cells. While Persantine has been a useful tool to generate preliminary data, it is a weak and reversible inhibitory of phosphodiesterase. Supplementing a diet of cardiovascular diseased patients with linoleic acid (the substrate for 13-HODE) also has been useful to demonstrate the benefit of elevating VW 13-HODE levels. However, that approach requires the patients to ingest a daily dose of 20 grams or more of linoleic acid-rich capsules, and the treatment is not without its unwanted side effects, including an increased caloric intake.

Surprising Results

During all of the years since I and all colleagues known to me who are working in the field, have concentrated on the idea of maintaining healthy endothelial cell function either by regulating the endogenous production of 13-HODE, avoiding factors which suppress its synthesis and/or providing agents containing linoleic acid which may enhance the synthesis of 13-HODE. We had thought that only trivial amounts of purified 13-HODE would reach the target site of the VW endothelium if administered orally, and would, therefore, be biologically inactive. Recently, the opportunity arose in which we could actually test that possibility. The results of those experiments have been of great surprise to us, and demonstrate that our initial point of view was wrong. Thus, we have found that orally-administered 13-HODE does reach its intended targets and is biologically active. In addition, we have identified suitable vehicles in which 13-HODE can be administered orally. And most amazingly, the beneficial effects of orally-administered 13-HODE are achieved with unexpectedly low doses.

The model in which we demonstrate the benefical effects of 13-HODE is a rabbit model of vascular response to VW injury. New Zealand white rabbits (half males/half females; 2.5 - 2.9 kg) were used throughout. Rabbits were treated with 100, 400 or 1000 µg/kg/day of purified 13-HODE suspended in corn oil or ethyl-eicosapentaenoic acid (EPA), or with an equivolume of either suspending vehicle (total volume 1 ml) for 7 days. Serial blood samples were collected before, during and after treatment to assess the levels of 13-HODE in plasma. On day 7, the treatments were stopped. At that time, the rabbits were anaesthetized with a combination of Atravet, Ketamine, Vetrepham and glycopyrolate, given

subcutaneously. Both carotid arteries of anaesthetized rabbits were isolated between 2 temporary ligatures, first by applying the proximal ligature, then allowing the blood to drain from the segment, and then applying the distal ligature. A 24 gauge angiocath attached to tubing filled with sterile saline and connected to a pressure manometer, was inserted into the isolated segment. Then the segment was filled with the saline to a pressure of 600 mm Hg which was maintained for 5 minutes. The pressure was relieved, and the angiocath and ligatures were removed, thereby allowing for blood flow restoration. Cessation of bleeding from the needle puncture site was achieved within 3 minutes without any manual manipulation. The incisions were sutured closed with 000 proline. The rabbits were injected intramuscularly with Temgesic to minimize any pain and with 12.5 mg Baytril as an antibiotic, and then allowed to recover. This injury procedure results in endothelial denudation and the exposure of a thrombogenic surface within 1 hour of restoration of blood flow, followed by SMC proliferation and intimal hyperplasia, which plateaus at ≈ 4 weeks and which is sustained for ≥12 weeks [Buchanan MR, Brister SJ: Inhibition of chronic vessel wall (re)stenosis with acute thrombin inhibition: relative effects of heparin and dermatan sulphate. *Thromb Res* 91: 157 - 167, 1998; Buchanan MR et al. Evidence for a conformational change of surface-bound thrombin that promotes vessel wall thrombogenicity: Selective and sustained inhibition by Intimatan but not by heparin. *Thromb Haemost* 81 (suppl): 1309, 1999].

Two, four or twelve weeks later, the rabbits were re-anaesthetized and their injured carotid arteries were again isolated. Segments of injured and uninjured carotid artery were

harvested and processed histologically to assess VW hyperplasia. Other VW segments and blood samples were collected and processed for VW and plasma 13-HODE levels.

All procedures were performed in accordance to the Canadian Animal Health and Welfare Act and approved by the McMaster University Animal Ethics Review Board [*Canadian Council of Animal Care: Guide to the care and use of experimental animals*, ED Olfert, BM Cross, AA McWilliam (eds), Bradda Printing Incorp, Vol 2, Ottawa, 1984].

Before that, we first had to identify an appropriate vehicle for 13-HODE. 13-HODE is a colourless or very pale yellow liquid. We found that it is stable without degradation in triglyceride oils, and also in esters. Thus, any triglyceride oil such as any vegetable oil, including corn, sunflower, safflower, soy, evening primrose, borage, coconut, palm, or other oil would be appropriate. We chose corn oil for some of our experiments because we had previously found that suspending linoleic acid in corn oil facilitated its selective uptake by the VW [Bertomeu M-C et al. Selective effects of dietary fats on vascular 13-HODE synthesis and platelet/vessel wall interactions. *Thromb Res* 59: 819-830, 1990]. We also chose marine fish oil, specifically marine menhaden or 97 % pure ethyl eicosapentaenoic (ethyl-EPA) since marine fish oils such as tuna, sardine or other oils also are suitable to maintain 13-HODE stability. Ethyl-EPA was of great interest therapeutically because EPA has many desirable actions other than on the VW endothelium such as the inhibition of platelet aggregation, the lowering of triglyceride levels and the attenuation of inflammation. However, it also can inhibit the formation of endogenous 13-HODE which might be a possible negative effect

(Miller CC, Ziboh, VA. Induction of epideral hyperproliferation by topical n-3 polyunsaturated fatty acids on guinea pig skin linked to decreased levels of 13-hydroxyoctadeca-dienoic acid (13-HODE). *J Invest Dermatol* 94: 353 - 358, 1990; Gimenez-Arnau A et al. Effects of linoleic acid supplements on atopic dermatitis. *Recent Adv Prost Thromboxane Leuko Res* 433: 285 - 289, 1997).

This inhibitory effect of EPA may help explain why the expected desirable cardiovascular effects of EPA have not been realized in practice. For example, a continuous course of EPA treatment failed to reduce restenosis after occluded coronary arteries had been opened by angioplasty (Cairns JA et al. Fish oils and low molecular weight heparin for the reduction of restenosis after percutaneous transluminal coronary angioplasty. *Circ* 94: 1553 - 1560, 1996).

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Results

Plasma 13-HODE Levels: There was a three-fold increase in the plasma 13-HODE levels after 7 days of treatment with 100 μg/kg/day of 13-HODE suspended in corn oil (Fig 1, upper panel). Increasing the 13-HODE dose to 1,000 μg/kg/day had no further effect. The plasma 13-HODE levels returned back to almost control levels within 14 to 21 days. Similar results were seen when the 13-HODE was suspended in ethyl-EPA (Fig 1, lower panel), although the absolute levels of plasma 13-HODE were lower in all treatment levels tested. Notwithstanding, there were no significant differences in the dose-related increases in plasma 13-HODE levels between the two suspending vehicle treatment groups.

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VW 13-HODE Levels: There also was a dose-related increase in VW wall 13-HODE levels

in the rabbits treated with 13-HODE suspended in corn oil and measured 28 days after stopping the treatment (Fig 2, upper panel) . These data indicated the VW 13-HODE levels remain elevated despite stopping the treatment. These data were very surprising to us. We had not expected orally administered 13-HODE to alter vascular wall 13-HODE at all, and we had not expected the short course of 13-HODE to maintain elevated levels detectable a whole month after stopping treatment. This may be due to the 13-HODE being incorporated into complex lipids as demonstrated by Fang et al (Fang X et al. 13-HODE incorporation and conversion to novel products by endothelial cells. *J Lipid Res* 40: 699 - 707, 1999).

Similar results were seen when the 13-HODE was suspended in ethyl-EPA (Fig 2, lower panel), but again, the absolute levels of VW 13-HODE were somewhat lower at all treatment levels tested. Since the VW 13-HODE levels were of course a measurement of both endogenous- and exogenously-derived 13-HODE, it is possible that the lower VW 13-HODE levels seen in the ethyl-EPA treated group reflect a suppression of endogenous 13-HODE synthesis as described by Miller and Ziboh (Miller CC, Ziboh, VA. Induction of epideral hyperproliferation by topical n-3 polyunsaturated fatty acids on guinea pig skin linked to decreased levels of 13-hydroxyoctadeca-dienoic acid (13-HODE). *J Invest Dermatol* 94: 353 - 358, 1990).

Biological Effects: In the initial studies with the purified 13-HODE, the marked increases in VW 13-HODE were associated with a significant decrease in intimal hyperplasia measured 2 and 4 weeks after injury. Thus, intimal hyperplasia in rabbits treated with 1,000 μg/kg/day of 13-HODE, was 8 and 16 % at 2 and 4 weeks respectively, compared to 18 and

38 % hyperplasia seen at 2 and 4 weeks, respectively in the placebo treated rabbits, $p < 0.002$ (Fig 3). Moreover, the intimal hyperplasia *regressed* in the 13-HODE treated animals such that intimal hyperplasia was barely detectable at 12 weeks, > 3%, $p < 0.01$. This is the first direct evidence demonstrating that purified 13-HODE inhibits VW intimal hyperplasia. It was interesting to note that the 13-HODE level in the cell-free plasmas of those animals not only was elevated significantly within 3 days of treatment, but remained elevated for 4 weeks despite our stopping the 13-HODE treatment after 7 days (Fig 4). These latter data suggest that orally administered 13-HODE is well absorbed, has a long half life, and can be monitored relatively easily in a clinical setting. These latter data also suggested to us that a markedly lower dose of 13-HODE could be as effective in preventing VW intimal hyperplasia.

Consistant with that possiblity and much to our surprise, we found that a 7 day course of 100 μg/kg/day of 13-HODE suspended in either corn oil or ethyl-EPA, significantly inhibited intimal VW hyperplasia, $p < 0.001$ (Fig 5). [For comparison, when patients ingest 3.2 gm of linoleic acid daily (20 capsules) for 30 days, VW 13-HODE levels only increases 2-fold (Buchanan MR, Brister SJ. Increasing vessel wall (VW) 13HODE metabolism with dietary fatty acid supplements in patients undergoing cardiac surgery decreases VW reactivity to platelets. *Can J Cardiol* 10 (Suppl C): 67C, 1994), and when rabbits were treated with Persantine in a dose comparable to that used clinically, VW 13-HODE only increased 30 to 50 % (Weber E et al. Relationship between vessel wall 13-HODE synthesis and vessel wall thrombogenicity following injury. Influence of salicylate and dipyridamole treatm nt. *Thromb Res* 57: 383-392, 1990. Platelet/injured VW

interactions were decreased > 2-fold in both cases].

These recent data are very exciting because they demonstrate that the effect; i.e. inhibition of intimal hyperplasia is achieved with an amazingly *low dose* of 13-HODE, and because hyperplasia *regresses*. We are not aware of any current antithrombotic treatment that has that capability. We also believe that this effect is achieved without impairing platelet function and coagulation, as based on our earlier studies (Weber E et al. Relationship between vessel wall 13-HODE synthesis and vessel wall thrombogenicity following injury. Influence of salicylate and dipyridamole treatment. *Thromb Res* 57: 383-392, 1990; Bertomeu M-C et al. Selective effects of dietary fats on vascular 13-HODE synthesis and platelet/vessel wall interactions. *Thromb Res* 59: 819-830, 1990; Brister et al. 13-HODE synthesis in internal mammary arteries and saphenous veins: Implications in cardiovascular surgery. *Adv Prost Thromb Leuko Res* 21: 667-670, 1990), and because we observed no detectable hemorrhagic defect in any of our experimental animals.

Experimental Conclusions: The following conclusions can be made from these experiments:

1) 13-HODE prevents VW hyperplasia effective when administered orally since the13-HODE will reach the vascular tissue and other tissues in concentrations which are biologically active and which restore VW biocompatability.

2) The doses of 13-HODE which are biologically active are surprisingly low.

3) Triglyceride and ester oils are appropriate vehicles for the 13-HODE.

4) The biological effects of 13-HODE on the vessel wall are highly desireable. These include both the prevention and treatment of vessel wall hyperplasia, as well as the

facilitation of vessel wall disease regression.

SUMMARY OF THE INVENTION AND MEDICINAL VALUE

As discussed earlier, 13-HODE is one of the factors which regulates vessel wall biocompatability, thereby attenuating untoward blood component/vessel wall interactions. It may do this in several ways, one of which probably is to reduce the expression and activation of the vitronectin receptor. In recent years, it has been found that abnormalities of vessel wall biocompatability are associated with a remarkable number of illnesses, including infections, cardiovascular problems of many types, as well as to problems relating to inflammation, fibrosis and loss of normal metabolic control (Vallance et al. Infection, inflammation and infarction: does acute endothelial dysfunction provide a link. *Lancet* 349: 1391 - 1392, 1997), and tumour cell metastasis. In all of these illnesses, vascular endothelial cells are activated, leading to the loss of the normal vascular permeability barrier, expression of leukocyte adhesion molecules, change in VW surface thromboreactivity, the production of a wide range of cytokines and the upregulation of HLA antigens (Hunt BJ, Jurd KM. Endothelial cell activation: a central pathophysiological process. *Br Med J* 316: 1328 - 1329). A wide range of illnesses may be caused or exacerbated by endothelial cell activation and damage. They include both Type I, Type II and the precurser Type II diabetes, syndrome X (Cosentino F, Lucher TF. Endothelial dysfunction in diabetes mellitus. *J Cardiovasc Pharmacol* 32 (suppl 3): 554 561, 1998; Steinberg AD. Endothelial function, insulin sensitivity and hypertension. *Circ* 96: 725 - 726, 1997),

many types of inflammatory disorders including rheumatoid arthritis and osteoarthritis and autoimmune diseases (Perretti M. Endogenous mediators that inhibit the leukocyte endothelium interaction. *TIPS* 18: 418 - 425, 1997), infections with bacteria and protozoa like malaria and sleeping sickness, and fungi which can generate endotoxins (Gerrity et al. Endothel-induced vascular endothelial injury and repair. *Exp Mol Path* 24: 59 - 69, 1976), sickle cell disease and related haemoglobins disorders (Lubin BH. Sickle cell disease and the endothelium. *New Engl J Med* 337: 1623 - 1625, 1997), kidney disease (Clausen et al. Endothelial haemostatic factors are associated with progression of urinary albumin excretion in clinically health subjects. *Clin Sci* 97: 37 - 43, 1999), inflammatory bowel disease (Binion DG et al. Acquired increases in leucocyte binding by intestinal; microvascular endothelium in inflammatory bowel disease. *Lancet* 352: 1742 - 1746, 1998) pregnancy hypertension and pre-eclampsia (Endresen MJR et al. Serum from pre-eclampsia women induces vascular adhesion molecule-1 expression on human endothelial cells in vitro. *Am J Obst Gynaecol* 179: 665 - 670, 1998), normal aging (Hashimoto M et al. Effect of aging on plasma membrane fluidity of rat aortic endothelial cells. Exp Gerontol. 34: 687 - 698, 1999), dementias (Iadecola C et al. SOD1 resolves cerebral endothelia dysfunction in mice overexpressing amyloid precursor protein. *Nature Neuroscience* 2: 157 - 161, 1999), retinal ischemia and age-related macular degeneration (Gidday JM, Zhu Y. Endothelium-dependent changes in retinal blood flow following ischemia. *Curr Eye Res* 17: 798 - 807, 1998; Wada M et al. Expression of vascular endothelial growth factor and its receptor mRNA in experimental choroidal neovascularisation. *Curr Eye Res* 18: 203 - 213, 1999), cancer and especially cancer metastasis and angiogenesis (Pinedo HM et al. Involvement of platelets in tumour angiogenesis? *Lancet* 352: 1775 - 1777, 1998; Shureigi I et al. Decreased 13-HODE levels and 15-lipoxygenase-1 expression in human colon cancers. *Carcinogenesis* 20: 1985 - 1995, 1999; Baron Ja et al. Venous thromboembolism and cancer. *Lancet* 351:

1077 - 1080, 1998; Hazelton D et al. Vascular endothelial growth factor levels in ovarian cyst fluid correlate with malignancy. *Clin Cancer Res* 5: 823 - 829, 1999), and in all types of cardiovascular diseases (Kanani PM et al. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocysteinemia in humans. *Circ* 100: 1161 - 1168, 1999), in hyperlipidemias and atherosclerosis of all types (Lefer DJ, Granger DN. Monocyte rolling in early atherogenesis. *Circ Res* 84: 1353 - 1355, 1999; Mombouli J-V. Endothelial dysfunction: from physiology to therapy. *J Mol Cellular Cardiol* 31: 61 - 74, 1999; Blann AD. Endothelial cell damage and the development or progression of atherosclerosis. *Clin Sci* 97: 119 - 121, 1999; Brown BG, Zhao X-Q. Importance of endothelial function in mediating the benefits of lipid-lowering therapy. *Am J Cardiol* 82: 49T - 52T, 1998; Freedman JE, Loscalzo J. Endothelial dysfunction and atherothrombotic occlusive disease. *Drugs* 54 (suppl 3): 41 - 50, 1997; Abe Y. Soluble cell adhesion molecules in hypertriglyceridemia and potential significance on monocyte adhesion. *Arterioscler Thromb Vasc Biol* 18: 723 - 731, 1998), transplantation (Labarrere CA et al. Endothelial activation of coronary disease in transplanted hearts. *JAMA* 278: 1169 - 1175, 1997), in pulmonary hypertension (Higenbottom TW, Laude EA. Endothelial dysfunction providing the basis for the treatment of pulmonary hypertension. *Chest* 114: 725 - 795, 1998), hypertension and heart failure (Boulanger CM. Secondary endothelial dysfunction: hypertension and heart failure. *J Mol Cellular Cardiol* 31: 39 - 49, 1999), and in Raynaud's syndrome (Edwards CM et al. Cardiovascular responses evoked by mild cool stimuli in primary Raynaud's disease: the role of endothelin. *Clin Sci* 96: 577 - 588, 1999). Endothelial function also is impaired by smoking (Motoyama T et al. Endothelial-dependent vasodilation in the brachial artery is impaired in smokers. *Am J Physiol* 273: H1644 - 50, 1997) in normal people who have a high fat meal (Plotnick GD et al. Effect of antioxidant vitamins on the transient impairment of endothelium-dependent brachial artery vasoactivity following a single high-fat meal. *JAMA* 278: 1682 - 1686, 1997), and

in apparently healthy people who had a low birth weight or who are at risk of cardiac disease (Goodfellow J et al. Endothelial function is impaired in fit young adults of low birth weight. *Cardiovasc Res* 40: 600 - 606, 1998; Ridker PM et al. Plasma concentrations of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *Lancet* 351: 88 - 92, 1998).

Thus, the regulation of endothelial function in a favourable direction by 13-HODE will have a desirable effect in a wide variety of diseases encompassing almost every aspect of medicine. Of particular interest is the fact that the administration of exogenous 13-HODE is able to compensate for any suppression of endogenous 13-HODE synthesis which can occur as a result of administration of omega-3 fatty acids such as alpha-linolenic acid, docoapentaenoic acid and particularly EPA and docosahexaenoic acid (DHA) (Miller and Ziboh, as above). EPA and DHA have many desirable actions but sometimes the clinical results of administering EPA and DHA are less favourable than expected, as in the case with attempts to prevent reocclusion after angioplasty (Cairns et al, as above). The co-administration of 13-HODE with EPA or DHA or other omega-3 fatty acids will therefore be of particular value.

13-HODE: CHARACTERISTIC AND SYNTHESIS

13-HODE is an oily liquid. Within the body 13-HODE can be metabolized as described earlier or transferred intact between various possible complex lipids including triglycerides, diaglycerides (diacylglycerols), monoglycerides, cholesterol esters and phospholipids of many different types. As a pharmaceutical, 13-HODE may be used as is,

or be dissolved in various carriers, or be incorporated into glyceride, ester, ph spholipid or other molecules with any appropriate carrier. Glycerides, esters of propane diol, ethyl esters and phospholipids to which 13-HODE is co-valently bound and any other molecules or vehicles which can release13-HODE in a biologically active form within the body all lie within this invention.

A real problem is presented by the fact that active daily doses of 13-HODE are in the 50 to 1,000 μg/kg/day range. The lower end of this range, 100 μg/kg/day, was shown to be highly biologically active in rabbits. That translates into a 7 mg/day dose for a 70 kg person. Since doses for humans are often considerably lower than doses for animals because of a weight/body surface area scaling, a daily dose of as little as 5 mg or less is really possible. Formulating an oily liquid is such small doses is a problem. It could be absorbed into tabletting materials and tabletted and coated, or micro-encapsulated by methods well known to those skilled in the art. However, we have found that the most convenient dosage form is to dissolve the 13-HODE into a triglyceride oil carrier. We have found that corn oil is a particularly useful diluent, and that 13-HODE can be readily and conveniently mixed with corn oil in a ratio, for example, from 1:3 to 1:100. Other triglyceride oils such as sunflower, safflower, cottonseed, rapeseed, olive, evening primrose, borage, fish body or fish liver oils may all be used for this purpose, similarly, esters are also appropriate. Particualrly useful are esters of fatty acids with 16-26 carbon atoms and one or more double bonds in the chain. We have found that the ethyl ester of EPA to be particularly appropriate, but equivalent

esters of fatty acids such as oleic, linoleic, alpha-linolenic, stearidonic, gamma-linolenic, dihomogammalinolenic, arachidonic, docosapentaenoic and DHA are all examples of esters which could be useful to carry the 13-HODE.

The preparations can then be further processed to give a final dosage form. The oils can be ingested directly, or appropriate antioxidants or flavours can be added, or they can be converted into palatable, flavoured emulsions by the use of emulsifying agents or flavouring well known to those skilled in the art. A particularly valuable dosage form is a soft gelatin or bonded hard gelatin capsule, or a similar capsule made with agar or other appropriate materials.

At present, the only readily available supplies of 13-HODE are in very expensive mg quantities for use as laboratory reagents. The properties of the pure material are shown in Figure 6. Larger quantities can now be prepared using soybean lipoxygenase or an enzyme of similar specificity. This enzyme metabolizes linoleic acid to 13-HODE. The reaction can be carried out in an appropriate vessel filled with a cooling and stirring system and pH, oxygen (dissolved oxygen content, DOC), and temperature probes. The reaction is first charged with 0.1 M borate buffer which is then chilled to below 100 ° C. The buffer is then purged with oxygen until the DOC reaches 100 %. Soybean lipoxygenase is then added at the rate of about 2500 U/litre and the mixture is stirred and regularly purged with oxygen to keep the DOC at 100 %. Octa-deca-9Z, 12Z-dienoic acid, dissolved 1/1 in ethanol is then added at a rate of about 10 g/litre. The reaction is then pressurized with an overblanket of oxygen, and vigorously stirred. The reaction is allowed to proceed, and monitored at 15

minute intervals by ultraviolet analysis and thin layer chromatography analysis to confirm the conversion of the linoleic acid to 13-HODE.

On completion of the reaction, the vessel is flushed with nitrogen and reduced by adding sodium borochydride at the rate of about 3.3 g/litre. On completion of the reduction process, the mixture is acidified to pH 6 with citric acid. Reverse phase silica (OD53) is then added and stirred and the mixture is allowed to continue to stir overnight under nitrogen at room temperature. The silica absorbs the 13-HODE, which is then recovered by filtering the silica, washing it with water, and then eluting out the product by multiple washing with acetonitrile. The solvent is then washed off and the crude oil is purified by column chromatography with diethyl ester and methylene chloride to yield pure 13-HODE as a viscous pale yellow oily substance. This material can then be formulated as discussed above.

EXAMPLES

1. 13-HODE in a ratio of between 1:3 and 1:100 or even up to 1:1000 with triglyceride oil, particularly corn oil. For example, 50 mg of 13-HODE could be mixed with 450 mg corn oil in a soft gelatin or bonded gelatin capsule, or 5 mg could be mixed with 100 mg of evening primrose oil or any other appropriate oil in similar types of capsules.

2. Compositions as in Example 1 but in which the oil is for direct administration as a liquid and is flavoured in an appropriate way, for example with lemon.

3. Compositions as in Example 1 but in which the oil is mixed with water to form a 5

to 40 % oil in water emulsion, using an appropriate emulsifier and appropriate flavourings.

4-6. Compositions as in Examples 1-3 but in which the oil is mixed with an ester, particularly an ethyl ester of a 16-18 carbon fatty acid with one or more double bonds. Ethyl oleate, ethyl-linolate, ethyl-EPA and ethyl-DHA are examples of appropriate carriers for 13-HODE.

7-12. As in Examples 1-6 but in which the 13-HODE is incorporated itself into a mono-, di- or tri-glyceride prior to mixing with the carrier.

13-18. As in Examples 1-6 but in which the 13-HODE is incorporated itself into the Sn1 or Sn2 positions of an appropriate phospholipid prior to mixing with the carrier.

19-24. As in Examples 1-6 but in which the 13-HODE is incorporated into any other appropriate carrier molecule which will allow the 13-HODE to be delivered to target sites where 13-HODE is biologically active, such as the vascular endothelium.

CLAIMS

1. Pharmaceutical compositions or formulations for the oral administration of 13-HODE either in its free form or incorporated into an appropriate carrier molecule.

2. As Claim 1 but where the maximum daily dose of 13-HODE is equal to or less than 100 mg.

3-4. As Claims 1-2 but where the 13-HODE is delivered dissolved in an appropriate mono-, di-, or triglyceride carrier.

5-7. As Claims 3-4 but where the carrier is corn oil.

8-9. As Claims 1-2 but where the 13-HODE is delivered in a carrier which is the ester of a fatty acid containing 16-26 carbon atoms and one or more double bonds.

10-11. As Claims 1- 9 but where the carrier is ethyl-EPA or ethyl-DHA.

12-21. As Claims 1-10 but where the formulation is used for the treatment or prevention of any human disease other than cancer.

22-31. As Claims 1-10 but where the formulation is used for the treatment or prevention of cancer or for the treatment or prevention of the metastatic spread of cancer.

32. A method of correcting the inhibition of endogenous 13-HODE synthesis by omega-3 fatty acids by incorporating 13-HODE into formulations of omega-3 fatty acids.

33. As Claim 32 but where the omega-3 fatty acids are EPA, DHA or their derivatives.

34. As Claim 32 but where the omega-3 fatty acids are in the form of ethyl-EPA or ethyl-DHA.

35-69. As Claims 1-34 but where the formulations are to be used for the treatment of

disorders where endothelial function is disordered.

70-104.As Claims 1-34, but where the formulations are to be used for the treatment of disorders associated with overactive protein kinases.